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A $^{99m}Tc(CO)_3$-labeled benzylguanidine with persistent heart uptake‡

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We describe the synthesis and biological evaluation of the cationic ^{99m}Tc–tricarbonyl complex *fac*-[$^{99m}Tc(CO)_3(\kappa^3$-L1)]$^+$ (Tc1) anchored by a pyrazole-diamine-methylbenzylguanidine-based ligand (L1), as potentially useful for myocardial imaging. The rhenium complex *fac*-[$Re(CO)_3(\kappa^3$-L1)]$^+$ (Re1) was prepared and characterized as a 'cold' surrogate of the radioactive complex. Cell uptake studies in a neuroblastoma cell line suggest that Tc1 uptake mechanism is related to the norepinephrine transporter (NET). Tissue distribution studies in CD1 mice showed that Tc1 presents high initial heart uptake and a slow washout from the heart (7.8 ± 1.3% injected dose per gram (ID/g), 30-min post-injection (p.i.); 6.3 ± 1.3% ID/g, 60-min p.i.), with heart to blood ratios of 11.8 and 9.0 at 30- and 60-min p.i., respectively. The uptake mechanism of Tc1 appears to be similar to that of metaiodobenzylguanidine (MIBG), as it can be reduced by coinjection with nonradioactive MIBG. The biodistribution profile of Tc2, where the benzylguanidine pharmacophore is absent, corroborates the fact that Tc1 does not accumulate in the heart by a simple diffusion mechanism but rather by a NET-mediated mechanism. The results confirm those obtained in the cell assays. Despite the persistent heart uptake found for Tc1, the high hepatic and renal uptake remains to be improved.

Keywords: metaiodobenzylguanidine; myocardial imaging; norepinephrine transporter; radiopharmaceuticals; rhenium; technetium-99m

Introduction

The most used radiotracers in the clinical setting for myocardial imaging are the cationic ^{99m}Tc-radiopharmaceuticals, ^{99m}Tc-sestamibi, and ^{99m}Tc-tetrofosmin. However, their pharmacokinetic profile is far from ideal. Indeed, they show high liver uptake, which can interfere in the analysis of cardiac imaging, particularly of the inferior left ventricular wall.[1–6] In the past years, intense research efforts have been focused on the development of novel cationic ^{99m}Tc-based probes with improved heart to background ratios. The most promising results have been obtained with the mixed-ligand $^{99m}Tc(V)$-nitrido complexes stabilized by bisphosphinoamine (PNP)-type ligands such as ^{99m}Tc-*N*-DBODC5, ^{99m}Tc-*N*-15C5, and ^{99m}Tc-*N*-MPO. Promising results were also attained with the $^{99m}Tc(CO)_3$-complex and ^{99m}Tc-15C5-PNP.[7–11] Recently, we have investigated the coordination chemistry of pyrazole-based ligands with the '$^{99m}Tc(CO)_3$' core, and we were able to identify a new class of organometallic complexes based on tri-methoxy-tris-pyrazolylmethane (TMEOP) potentially useful for myocardial imaging.[12–15] Among these complexes, *fac*-[$^{99m}Tc(CO)_3\{\kappa^3$-HC[3,4,5-$(CH_3OCH_2)_3$pz]$_3\}$]$^+$ (^{99m}Tc-tri-methoxy-tris-pyrazolylmethane (TMEOP)) showed a high heart uptake and a biodistribution profile suitable for myocardial imaging.[13,14] Their heart uptake mechanism is similar to that of other reported monocationic ^{99m}Tc-based cardiac agents and is associated with accumulation in the mitochondria of myocytes.[15–19]

Another family of radiotracers successfully applied to myocardial imaging is represented by radioiodinated metaiodobenzylguanidine (MIBG), an analog of norepinephrine, which is used for scintigraphic imaging studies of cardiac sympathetic innervations.[20,21] MIBG is actively transported into presynaptic sympathetic nerve terminals by the norepinephrine transporter (NET), and its uptake matches the density and integrity of sympathetic neurons.[22–25] Radioiodinated MIBG has been also widely used for the diagnosis of neuroendocrine tumors.[26,27] Radiohalogenated analogs of MIBG such as $^{77/76}$Bromo-labeled and ^{18}Fluoro-labeled guanidines, as well as ^{11}C-labeled guanidines, have been also explored as potential myocardial imaging agents.[26,28–32] With regard to the targeting of the NET with $^{99m}Tc(V)$-complexes for single photon emission computed tomography imaging of sympathetic neurons, Kung *et al*. have introduced a set of $^{99m}Tc(V)$-labeled MIBG derivatives, containing chelating groups of the N_2S_2 type, which compare well with radioiodinated MIBG in terms of heart uptake.[33] The numerous advantages of the '$M(CO)_3$' core for radiopharmaceutical applications prompted us to develop a $^{99m}Tc(I)$-MIBG derivative potentially useful as a heart imaging agent.[34–38] In this context, we designed a novel bifunctional chelator (**L1**), which combines a tridentate pyrazolyl-diamine chelating backbone for metal stabilization and a pendant

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benzylguanidine moiety for probing the NET. The conjugate **L1** allowed the synthesis of the MIBG derivative *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L1**)]$^+$ (**Tc1**), whose biological properties were assessed in a neuroblastoma cell line as well as in CD1 mice.

Experimental

General procedures and materials

All chemicals and solvents were of reagent grade and were used without purification unless stated otherwise. The Boc-protected precursors *tert*-butyl (2-{[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}ethyl)carbamate (**1**) and [Re(CO)$_3$(H$_2$O)$_3$]Br were prepared according to published methods.[39,40] All other chemicals not specified previously were purchased from Aldrich. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Varian Unity 300-MHz spectrometer. ^{1}H and ^{13}C chemical shifts were referenced with the residual solvent resonances relatively to tetramethylsilane. The spectra were assigned with the help of 2D experiments (^{1}H–^{1}H correlation spectroscopy, and ^{1}H–^{13}C heteronuclear single quantum coherence). Assignments of the ^{1}H and ^{13}C NMR resonances are given in accordance with the identification system shown in Scheme 1. Infrared (IR) spectra were recorded as KBr pellets on a Bruker Tensor 27 spectrometer. Compounds were characterized by electrospray ionization mass spectrometry (ESI-MS) using a quadrupole ion trap mass spectrometer (QITMS) instrument. Aliquots of ~5 mg of pure compounds ≥95% ascertained by reversed phase HPLC (RP-HPLC) were lyophilized in eppendorf tubes and used for radioactive labeling and *in vitro* studies. Na[^{99m}TcO$_4$] was eluted from a ^{99}Mo/^{99m}Tc generator using 0.9% saline. HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV ultraviolet–visible (UV/Vis) and to a Berthold LB 509 radiometric detector, using an analytic Macherey–Nagel C18 reversed-phase column (Nucleosil 100-5, 250 × 3 mm) with a flow rate of 0.5 mL/min. Purification of the inactive compounds was achieved on a semipreparative Macherey–Nagel C18 reversed-phase column (Nucleosil 100-7, 250 × 8 mm) with a flow rate of 2.0 mL/min. UV detection: 220 or 254 nm. Eluents: A = aqueous 0.1% CF_3COOH and B = MeOH. Gradient of *method 1*: *t* = 0–3 min, 0% B; 3–3.1 min, 0 → 25% B; 3.1–9 min, 25% B; 9–9.1 min, 25 → 34% B; 9.1–20 min, 34 → 100% B; 20–25 min, 100% B; 25–25.1 min, 100 → 0% B; and 25.1–30 min, 0% B. Gradient of *method 2*: *t* = 0–5 min, 10% MeOH; 5–30 min, 10 → 100% MeOH; 30–34 min, 100% MeOH; 34–35 min, 100 → 10% MeOH; and 35–40 min, 10% MeOH.

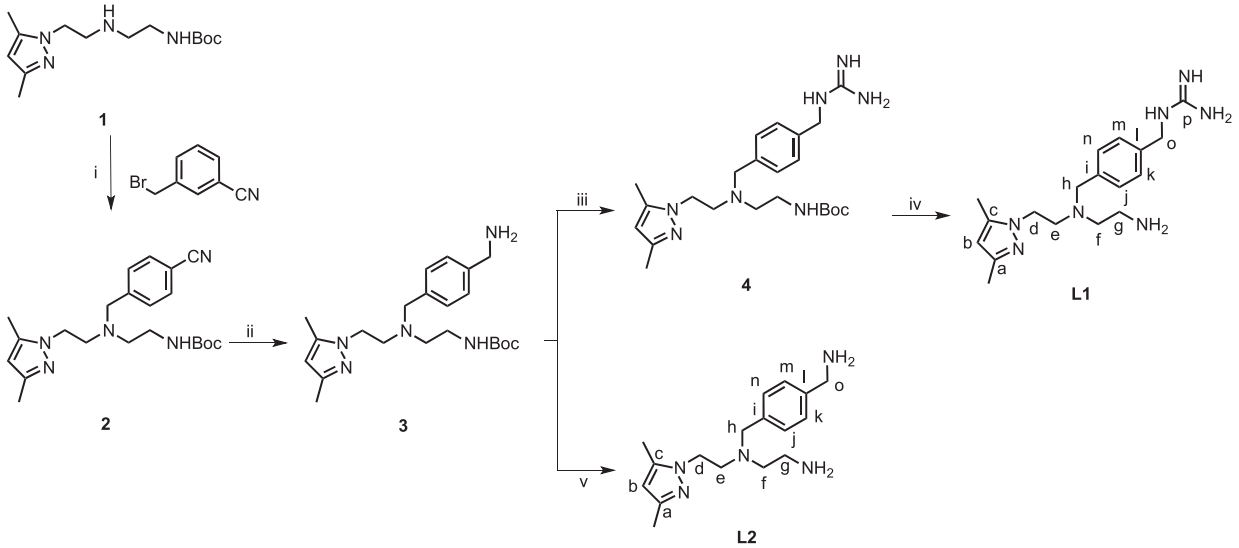
Synthetic procedures

Preparation of tert-butyl 2-((4-cyanobenzyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)-ethylcarbamate (2)

A solution of **1** (0.600 g, 2.125 mmol), 3-(bromomethyl)benzonitrile (0.833 g, 4.250 mmol), and K_2CO_3 (0.587 g, 4.250 mmol) in acetonitrile (ACN) (35 mL) was refluxed for 24 h. After the reaction, the solution was filtered and the solvent evaporated to give an oily residue, which was purified by silica gel column chromatography using a gradient of EtOAc (0 → 100%) in hexane. The fractions containing **2** were collected, and the compound was obtained as colorless oil after evaporation of the solvent. Yield: 55.6% (0.470 g, 1.183 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.45 (2H, m, $CH^{m/k}$), 7.29 (2H, m, $CH^{n/j}$), 6.00 (1H, br m, N*H*Boc), 5.82 (1H, s, CH^b), 4.00 (2H, t, *J* = 7.8 Hz, CH_2^d), 3.55 (2H, s, CH_2^h), 3.20 (2H, m, CH_2^e), 2.70 (2H, t, *J* = 7.6 Hz, CH_2^f), 2.61 (2H, t, *J* = 7.6 Hz, CH_2^g), 2.28 (3H, s, $CH_3^{a/c}$), 1.98 (3H, s, $CH_3^{a/c}$), and 1.44 (9H, s, $C(CH_3)_3$). ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ_C (ppm) 156.6 (C=O, Boc), 147.5 (C^c), 141.2 (C^a), 138.7 (C^i), 133.0 ($C^{k/m}$), 132.0 ($C^{k/m}$), 130.8 ($C^{j/n}$), 129.1 ($C^{j/n}$), 119.12 (C^l), 112.6 ($C^{C\equiv N}$), 105.7 (C^b), 79.1 ($\underline{C}(CH_3)_3$, 58.0 (C^h), 53.4 (C^e), 53.3 (C^f), 46.7 (C^d), 38.6 (C^g), 28.7 ($C(\underline{C}H_3)_3$, 13.6 ($CH_3^{a/c}$), and 10.9 ($CH_3^{a/c}$). IR (KBr, cm^{-1}): 2229vw ν(C≡N), 1711 m, and 1175 m.

Preparation of tert-butyl 2-((4-(aminomethyl)benzyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)-amino)ethylcarbamate (3)

$NiCl_2 \cdot 6H_2O$ (0.023 g, 0.096 mmol) was added to a solution of **2** (0.382 g, 0.961 mmol) in MeOH (10 mL) cooled with an ice bath to give a green solution. $NaBH_4$ (0.290 g, 7.688 mmol) was added in portions with stirring, and the resulting purple mixture was stirred overnight at 20°C. The solvent was evaporated and the residue partitioned between EtOAc and a saturated $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$. Removal of organic solvent gave a colorless residue. Compound **3** was used without any further purification. Yield: 79.5% (0.307 g, 0.764 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.17–6.98 (4H, m, $CH^{j/k/m/n}$), 5.75 (1H, s, CH^b), 5.50 (2H, br m, N*H*$_2$), 3.98 (2H, s, CH_2^o), 3.78 (2H, m, CH_2^d), 3.56 (2H, s, CH_2^h), 3.14 (2H, m, CH_2^e), 2.76 (2H, t, *J* = 7.7 Hz, CH_2^f), 2.56 (2H, t, *J* = 7.7 Hz, CH_2^g), 2.23 (3H, s, $CH_3^{a/c}$), 2.02 (3H, s, $CH_3^{a/c}$), and 1.42 (9H, s, $C(CH_3)_3$). ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ_C (ppm) 156.5 (C = O, Boc), 147.5 (C^c), 139.5 (C^a), 139.2 (C^l), 128.8 ($C^{j/n}$), 128.7 ($C^{j/n}$), 127.3 ($C^{k/m}$), 127.4 ($C^{k/m}$), 125.9 (C^i), 105.2 (C^b), 79.1 ($\underline{C}(CH_3)_3$, overlapped with $CDCl_3$), 58.9 (C^e), 53.4 (C^f), 53.3 (C^d), 47.0 ($C^{o/h}$), 45.0 ($C^{o/h}$), 38.6 (C^g), 28.7 ($C(\underline{C}H_3)_3$), 13.7 ($CH_3^{a/c}$), and 11.1 ($CH_3^{a/c}$). IR (KBr, cm^{-1}): 1713 m and 1176 m.



Scheme 1. Synthesis of **L1** and **L2**. (i) K_2CO_3, KI, ACN, reflux, 24 h, 55.6%; (ii) $NaBH_4$, $NiCl_2$, MeOH, r.t., overnight, 79.5%; (iii) 1H-pyrazole-1-carboximidamide, DIPEA, DMF, overnight, 76.4%; (iv) TFA, CH_2Cl_2, r.t., 2 h, 38.8%; and (v) TFA, CH_2Cl_2, r.t., 2 h, 55.5% (identification system for NMR spectra assignment is displayed for **L1** and **L2**).

Preparation of tert-butyl 2-((2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl) (4-(guanidinomethyl)-benzyl)amino)ethylcarbamate (4)

A solution of **3** (0.065 g, 0.162 mmol), 1H-pyrazole-1-carboximidamide (0.036 g, 0.243 mmol), and *N,N'*-diisopropylethylamine (DIPEA) (0.031 g, 0.243 mmol) in dimethylformamide (DMF) was stirred for 48 h. After the reaction, the DMF was evaporated to give an oily residue, which was purified by silica gel column chromatography using a gradient of MeOH (0 → 100%) in $CHCl_3$. The fractions containing **4** were collected, and the compound was obtained as pale yellow oil after evaporation of the elution solvents. Yield: 76.4% (0.055 g, 0.123 mmol). ^{1}H-NMR (300 MHz, CD_3OD): δ_H (ppm) 7.29–7.10 (4H, m, $CH^{j/k/m/n}$), 5.80 (1H, s, CH^b), 4.34 (2H, s, CH_2^o), 4.00 (2H, t, J = 7.7 Hz, CH_2^d), 3.33 (2H, s, CH_2^h), 3.07 (2H, m, CH_2^e), 2.77 (2H, t, J = 7.6 Hz, CH_2^f), 2.58 (2H, t, J = 7.6 Hz, CH_2^g), 2.15 (3H, s, $CH_3^{a/c}$), 2.13 (3H, s, $CH_3^{a/c}$), and 1.31 (9H, s, $C(CH_3)_3$). ^{13}C-NMR (75.5 MHz, CD_3OD): δ_c (ppm) 157.5 (p), 157.1 (C=O, Boc), 154.0 (C^c), 144.0 (C^a), 140.1 (C^l), 129.3 ($C^{j/n}$), 128.7 ($C^{j/n}$), 128.3 ($C^{k/m}$), 127.6 ($C^{k/m}$), 125.9 (C^i), 104.8 (C^b), 78.8 ($\underline{C}(CH_3)_3$), 58.5 (C^e), 53.6 (C^f), 53.3 (C^d), 46.6 ($C^{o/h}$), 44.9 ($C^{o/h}$), 38.2 (C^g), 27.6 ($C(\underline{C}H_3)_3$), 11.7 ($CH_3^{a/c}$), and 9.9 ($CH_3^{a/c}$). IR (KBr, cm^{-1}): 1700 s and 1169 m. Analytic HPLC (R_t): 18.2 min (λ = 220 nm; method 1).

Preparation of 1-(4-(((2-aminoethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl) ethyl)amino)methyl)-benzyl)guanidine (L1)

Treatment of **4** (0.045 g, 0.101 mmol) with CH_2Cl_2/TFA (1 mL/2 mL) gave a colorless oil corresponding to **L1** after purification by semipreparative RP-HPLC. Yield: 37.6% (0.022 g, 0.038 mmol, calcd. for $C_{18}H_{31}N_7 \cdot 2\,CF_3COO^-$). ^{1}H-NMR (300 MHz, CD_3OD): δ_H (ppm) 7.24–7.03 (4H, m, $CH^{j/k/m/n}$), 5.86 (1H, s, CH^b), 4.30 (2H, s, CH_2^o) 4.08 (2H, t, J = 7.7 Hz, CH_2^d), 3.71 (2H, s, CH_2^h), 3.04 (2H, m, CH_2^e), 2.88 (2H, t, J = 7.7 Hz, CH_2^f), 2.80 (2H, t, J = 7.7 Hz, CH_2^g), 2.20 (3H, s, $CH_3^{a/c}$), and 2.00 (3H, s, $CH_3^{a/c}$). ^{13}C-NMR (75.5 MHz, CD_3OD): δ_c (ppm) 157.5 (C^p), 147.0 (C^c), 140.4 (C^a), 138.7 (C^l), 136.6 ($C^{j/n}$), 129.1 ($C^{j/n}$), 128.3 ($C^{k/m}$), 127.8 ($C^{k/m}$), 126.4 (C^i), 105.3 (C^b), 58.6 (C^e), 52.6 ($C^{f/g}$), 50.8 ($C^{o/h}$), 45.7 (C^d), 44.8 ($C^{o/h}$), 37.1 ($C^{f/g}$), 11.8 ($CH_3^{a/c}$), and 9.4 ($CH_3^{a/c}$). ESI-MS (+) (*m/z*): 344.2 $[M+H]^+$, calcd. for $C_{18}H_{29}N_7$ = 343.2. Analytic HPLC (R_t): 15.2 min (λ = 220 nm; method 1). IR (KBr, cm^{-1}): 3600 m, 1678 vs, 1205 m and 1141 w.

*Preparation of N^1-(4-(aminomethyl)benzyl)-N^1-(2-(3,5-dimethyl-1H-*pyrazol-1-yl)ethyl)ethane-1,2-diamine (*L2*)

Treatment of **3** (0.055 g, 0.137 mmol) with CH_2Cl_2/TFA (1 mL/2 mL) gave a colorless oil corresponding to **L2** after purification by semipreparative RP-HPLC. Yield: 55.5% (0.040 g, 0.076 mmol, calcd. for $C_{17}H_{29}N_5 \cdot 2\,CF_3COO^-$). ^{1}H-NMR (300 MHz, D_2O): δ_H (ppm) 7.26–6.99 (4H, m, $CH^{j/k/m/n}$), 5.82 (1H, s, CH^b), 4.17 (2H, t, J = 7.7 Hz, CH_2^d), 4.12 (2H, s, CH_2^h), 4.05 (2H, s, CH_2^o), 3.17 (6H, m, CH_2^{e+f+g}), 2.02 (3H, s, $CH_3^{a/c}$), and 2.00 (3H, s, $CH_3^{a/c}$). ^{13}C-NMR (75.5 MHz, D_2O): δ_c (ppm) 148.3 (C^c), 141.5 (C^a), 139.8 (C^l), 136.1 ($C^{j/n}$), 130.5 ($C^{j/n}$), 129.4 ($C^{k/m}$), 129.1 ($C^{k/m}$), 126.0 (C^i), 105.7 (C^b), 57.8 (C^e), 52.3 ($C^{f/g}$), 52.0 ($C^{o/h}$), 46.24 (C^d), 46.20 ($C^{o/h}$), 37.3 ($C^{f/g}$), 12.2 ($CH_3^{a/c}$), and 9.8 ($CH_3^{a/c}$). ESI-MS (+) (*m/z*): 302.3 $[M+H]^+$, calcd. for $C_{17}H_{27}N_5$ = 301.2. Analytic HPLC (R_t): 14.6 min (λ = 220 nm; method 1). IR (KBr, cm^{-1}): 3360 m, 1684 vs, 1640 sh, 1205 vs, and 1139 s.

General procedure for the preparation of Tc(I) complexes of the type *fac*-[Tc(CO)$_3$(κ^3-L)] (Tc1, L = L1; Tc2, L = L2).

The radioactive precursor *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ was prepared by addition of Na[^{99m}TcO$_4$] to an IsoLink® kit (Mallinckrodt-Covidean, Inc.) following a described procedure [38]. In a nitrogen-purged glass vial, 900 µL of *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (1–2 mCi) in saline pH 7.4 was added to 100 µL of a 10^{-3} or 10^{-4} M aqueous solution of the compounds **L1** or **L2**. The reaction mixture was then heated to 100°C for 30 min, cooled on an ice bath, and the final solution was analyzed by RP-HPLC. Retention times (method 2): 5.0 min for *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$, 23.1 min for **Tc1**, and 20.6 min for **Tc2**.

General procedure for the preparation of Re(I) complexes of the type *fac*-[Re(CO)$_3$(κ^3-L)] (Re1, L = L1; Re2, L = L2).

[Re(CO)$_3$(H$_2$O)$_3$]Br was reacted with 1.2 equivalents of the compounds **L1** and **L2** in refluxing H_2O for 18 h. After reacting, the solvent was removed under vacuum, and the resulting residue was dissolved in water and purified by semipreparative RP-HPLC (method 2).

Synthesis of *fac*-[Re(CO)$_3$(κ^3-L1)] (Re1 · 2TFA)

Starting from 0.045 g (0.078 mmol) of **L1** · 2TFA, a colorless oil formulated as **Re1** was obtained. Yield: 76.2% (0.050 g, 0.059 mmol, calcd. for $C_{21}H_{30}N_7O_3Re \cdot 2CF_3COO^-$). ^{1}H-NMR (300 MHz, CD_3OD): δ_H (ppm) 7.42–7.27 (4H, m, $CH^{j/k/m/n}$), 6.00 (1H, s, CH^b), 5.23 (1H, m, NH), 4.48 (2H, dd, J = 7.2 Hz, CH_2^h), 4.33 (2H, s, CH_2^o), 4.13 (2H, m, CH_2^d), 4.13 (2H, m, $CH_2^{d'}$), 3.74 (1H, m, NH), 3.20 (1H, m, CH_2^e), 3.00 (2H, m, CH_2^g), 2.56 (1H, m, $CH_2^{e'}$), 2.45–2.33 (2H, m, $CH_2^{f,f'}$), 2.25 (3H, s, $CH_3^{a/c}$), and 2.21 (3H, s, $CH_3^{a/c}$). ^{13}C-NMR (75.5 MHz, CD_3OD): δ_c (ppm) 194.6 (C≡O), 194.0 (C≡O), 193.0 (C≡O), 157.1 (C^p), 154.1 (C^c), 144.4 (C^a), 137.0 (C^l), 132.6 ($C^{j/n}$), 132.2 ($C^{j/n}$), 131.2 ($C^{k/m}$), 129.3 ($C^{k/m}$), 128.0 (C^i), 105.0 (C^b), 69.2 (C^f), 61.1 (C^h), 51.3 (C^e), 46.1 (C^d), 44.4 (C^g), 41.8 (C^o), 14.4 ($CH_3^{a/c}$), and 10.8 ($CH_3^{a/c}$). ESI-MS (+) (*m/z*): 614.2 $[M]^+$, calcd. for $C_{21}H_{29}N_7O_3Re$ = 614.2. IR (KBr, cm^{-1}): 2028 and 1911 ν(C≡O). HPLC (R_t): 22.4 min (λ = 254 nm, method 2).

Synthesis of *fac*-[Re(CO)$_3$(κ^3-L2)] (Re2 · 2TFA)

Starting from 0.025 g (0.047 mmol) of **L2** · 2TFA, a colorless oil formulated as **Re2** was obtained. Yield: 78.7% (0.030 g, 0.037 mmol, calcd. for $C_{20}H_{28}N_5O_3Re \cdot 2CF_3COO^-$). ^{1}H-NMR (300 MHz, CD_3OD): δ_H (ppm) 7.46–7.20 (4H, m, $CH^{j/k/m/n}$), 6.08 (1H, s, CH^b), 5.20 (1H, m, NH), 4.46 (2H, dd, J = 7.3 Hz, CH_2^h), 4.33 (1H, m, CH_2^d), 4.13 (2H, s, CH_2^o), 3.80 (1H, m, NH), 3.25–3.07 (4H, m, $CH_2^{d'}$, CH_2^e, NH), 2.56 (1H, m, $CH_2^{e'}$), 2.45–2.33 (2H, m, CH_2^g), 2.33 (3H, s, $CH_3^{a/c}$), and 2.20 (3H, s, $CH_3^{a/c}$). ^{13}C-NMR (75.5 MHz, CD_3OD): δ_c (ppm) 195.9 (C≡O), 194.6 (C≡O), 194.3 (C≡O), 156.4 (C^c), 144.4 (C^a), 133.6 (C^l), 133.3 ($C^{j/n}$), 133.2 ($C^{j/n}$), 133.0 ($C^{k/m}$), 132.6 ($C^{k/m}$), 132.5 (C^i), 105.0 (C^b), 68.6 (C^f), 60.8 (C^h), 50.5 (C^e), 45.9 (C^d), 43.0 (C^g), 41.8 (C^o), 15.4 ($CH_3^{a/c}$), and 10.8 ($CH_3^{a/c}$). ESI-MS (+) (*m/z*): 572.2 $[M]^+$, calcd. for $C_{20}H_{27}N_5O_3Re$ = 572.2. HPLC (R_t): 19.8 min (λ = 254 nm, method 2).

Determination of the partition coefficient (Log P$_{o/w}$)

Evaluated by the 'shake flask' method,[41] **Tc1** was added to a mixture of octanol (1 mL) and 0.1 M PBS pH = 7.4 (1 mL), previously saturated in each other by stirring the mixture. This mixture was vortexed and centrifuged (3000 *g*, 10 min, room temperature) to allow phase separation. Aliquots of both octanol and PBS were counted in a gamma counter. The partition coefficient (P$_{o/w}$) was calculated by dividing the counts in the octanol phase by those in the buffer, and the results were expressed as Log P$_{o/w}$. **Tc1**, Log P$_{o/w}$ = −0.763 ± 0.021; **Tc2**, Log P$_{o/w}$ = −0.889 ± 0.447.

Cell assays

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle medium containing GlutaMAX I supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibiotic solution (all from Gibco, Invitrogen, UK). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, with the medium changed every 2 days. The cells were adherent in monolayers and, when confluent, were harvested from the cell culture flasks with trypsin–EDTA (Gibco, Invitrogen, UK) and seeded into 24-well plates [(2–4) × 10^5 cells/per well in 500 µL of medium] and incubated for 24 h at 37°C. On the day of the experiment, the medium was discarded and replaced by fresh medium that contained the radioactive compound. The cells were exposed to **Tc1** for 15, 30 min, 1, and 2 h at 37°C. Incubation was terminated by washing the cells with ice-cold assay medium, washed twice with cold PBS, and subsequently, the cells were lysed by 10-min incubation with 1 N NaOH at 37°C. The percent of cell-associated radioactivity as a function of time was determined by counting in a

Berthold LB 2111 gamma counting system. Uptake studies were carried out using at least four wells for each data point. Blocking studies were performed by preincubating cells with DMI (50 μM) for 30 min.

Biodistribution studies in mice

All animal experiments were performed in compliance with Portuguese Law and European Directives (Portaria 1131/97, Decreto-Lei nº 129/92 de 6 de Julho e 197/96 de 16 de Outubro and 86/609/CEE) regarding ethics, care, and protection of animals used for experimental and other scientific proposes. The animals were housed in a temperature (22–23°C) and humidity (45–65%) controlled room with a 12-h light/12-h dark schedule. Biodistribution of the ^{99m}Tc complexes **Tc1** and **Tc2** was evaluated in groups of 3–4 CD1 female mice (Charles River outbred strain, from IFFA CREDO, Barcelona, Spain). Mice with 22–29-g weight were intravenously injected with 100 μL of each radiolabeled complex (in PBS) (60–90 μCi) via the tail vein, and the animals were kept in normal diet *ad libitum*. Blocking studies were carried out by coinjecting nonradioactive MIBG (1 mg/kg) together with **Tc1**. The effect of L-lysine on nonspecific kidney uptake of radiocomplex **Tc1** was checked up by coinjection with L-Lys (15 mg/50 μl saline) in order to block the cationic transporters on renal tubules. Animals were sacrificed by cervical dislocation at 2, 30 and 60 min after injection. The administered dose and the radioactivity in the sacrificed animals were measured with a dose calibrator (Curiemeter IGC-3, Aloka, Tokyo, Japan or Carpintec CRC-15W, Ramsey, USA). The difference in radioactivity between the injected animal and the sacrificed animal was assumed to be because of excretion. Tissues and organs of interest were dissected, rinsed to remove excess blood, weighted, and their radioactivity was measured using the dose calibrator or a γ-counter. The uptake in the tissues or organs was calculated and expressed as percent injected dose per gram of tissue or organ (% ID/g). For blood, bone, and muscle, total activity was estimated assuming that they represent 6, 10, and 40, of the total body weight, respectively. Blood samples were taken by cardiac puncture at sacrifice. The blood was then centrifuged, and the serum was separated and treated for HPLC analysis. Urine was also collected and pooled together at sacrifice time.

In vivo stability

The *in vivo* stability of **Tc1** was evaluated by RP-HPLC analysis of mice urine and blood samples. The urine was collected at the sacrificed time, centrifuged at 3000 *g* for 15 min and analyzed by RP-HPLC. The blood was collected at the sacrificed time, centrifuged at 3000 *g* for 15 min at 4°C, and the serum was separated. One hundred microliter aliquots of serum were sampled and treated with 200 μl of ethanol to precipitate the proteins. Samples were then centrifuged at 3000 *g* for 15 min at 4°C. The supernatant was analyzed by RP-HPLC.

Results and discussion

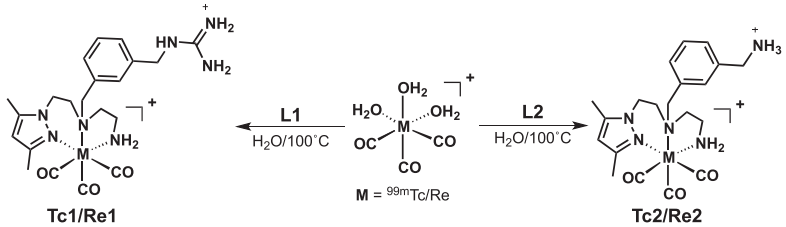
The synthetic pathway for the preparation of conjugate **L1** is displayed in Scheme 1. Direct alkylation of precursor **1** with 3-(bromomethyl)benzonitrile, followed by reduction of the resulting intermediate **2** with $NaBH_4/NiCl_2$ gave **3**, which upon reaction with 1*H*-pyrazole-1-carboximidamide afforded **4** (Scheme 1). Boc-deprotection of **4** and **3** under standard conditions afforded **L1** and **L2**, respectively. The latter ligand, where the guanidine moiety is absent, was synthesized with the aim of preparing the complex *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L2**)]$^+$, which will be used as a negative control in the biodistribution studies. All compounds were fully characterized by ESI-MS and ^{1}H/^{13}C-NMR and IR spectroscopy.

The reduction of the C≡N group in **2** was confirmed by ^{1}H-NMR and IR spectroscopy. Indeed, the absence of the absorption band at 2229 cm^{-1} in the IR spectrum, assigned to the stretching vibrations of the C≡N group, and the appearance of two new resonances at δ 5.50 (2H, br m) and 3.98 (2H, s) in the ^{1}H-NMR spectrum ($CDCl_3$), assigned to the additional amine and CH_2 groups, respectively, indicated that the formation of **3**. ^{1}H-NMR spectra of **L1** (CD_3OD) and **L2** (D_2O) showed resonances for the pyrazole-diamine backbone (**L1**: δ 5.86, 1H^b; δ 4.08, 2H^d; δ 3.04, 2H^e; δ 2.88, 2H^f; δ 2.80, 2H^g; δ 2.20, 3H^{CH3pz}; δ 2.00, 3H^{CH3pz} and **L2**: δ 5.82, 1H^b; δ 4.17, 2H^d; 3.17, 6H^{e+f+g}; 2.02, 3H^{CH3pz}; δ 2.00, 3H^{CH3pz}) and for the methyl-benzyl-guanidine (δ 7.24–7.03, 4H^{ar}; δ 4.30, 2H^o; δ 3.71, 2H^h) or methyl-benzyl-amine arms (δ 7.26–6.99, 4H^{ar}; δ 4.12, 2H^h; δ 4.05, 2H^o). The ^{13}C-NMR spectra of **L1** and **L2** display all the expected resonances, in particular, the presence of the carbon atom of the guanidine moiety in **L1** (δ 157.5), which was not present in **L2**.

The complexes *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L1**)]$^+$ (**Tc1**) and *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L2**)]$^+$ (**Tc2**) were synthesized by reaction of **L1** and **L2**, respectively, with the organometallic precursor *fac*-[^{99m}Tc(CO)$_3$(H_2O)$_3$]$^+$ (Scheme 2), which was prepared using an IsoLink® kit (Mallinckrodt, Covidien). The kit formulation, available for research purposes, contains boranocarbonate ([H_3BCO_2]$^{2-}$), Na/K tartrate, sodium tetraborate decahydrate, and sodium carbonate. The boranocarbonate reduces the Tc(VII) and acts simultaneously as a CO source, through mechanisms not yet fully understood.

Both complexes were obtained in high radiochemical yield (>95%) and high radiochemical purity (>95%), and the chemical identity was established by comparing their γ-traces (HPLC radiochromatograms) with the UV/Vis traces of the respective Re surrogates **Re1** and **Re2**. For the sake of example, we present the traces of the match pair **Tc1**/**Re** in Figure 1.

The complexes **Re1** and **Re2** were prepared by reaction of **L1** and **L2** with *fac*-[$Re(CO)_3(H_2O)_3$]Br (Scheme 2), respectively. The use of Re complexes to identify the molecular structure of the ^{99m}Tc congeners, by means of HPLC comparison, is a common practice in radiopharmaceutical chemistry because of the similar physicochemical properties of these group 7 elements. The complexes were fully characterized by the common spectroscopic techniques, and the data collected were consistent with a κ^3-N_3 coordination mode for the pyrazolyl-diamine chelator, as found in other similar Re(I) complexes already described by our group.[42–45]



Scheme 2. Preparation of *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L1**)]$^+$ (**Tc1**) and *fac*-[^{99m}Tc(CO)$_3$(κ^3-**L2**)]$^+$ (**Tc2**).

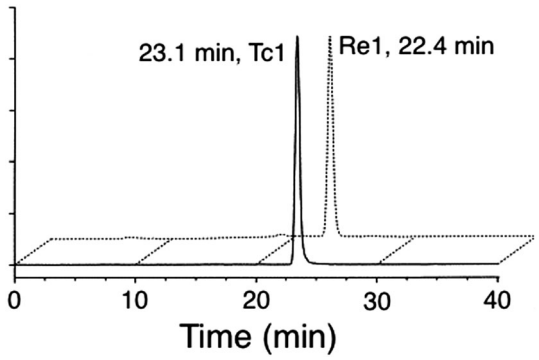


Figure 1. HPLC chromatograms of coinjected rhenium complex (**Re1**) (UV detection) and ^{99m}Tc complex (**Tc1**) (γ detection).

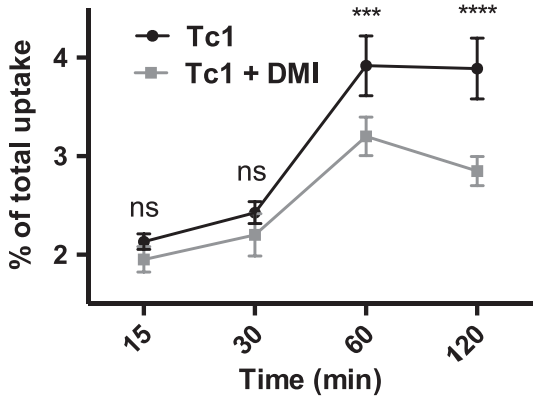


Figure 2. Uptake of **Tc1** in SH-SY5Y neuroblastoma cells as a function of incubation time (**Tc1**) and in the presence of desipramine (**Tc1** + DMI). All results are presented as the mean ± SEM ($n = 4$). The data shown were from a set of representative experiments. *** ($p = 0.0006$) and **** ($p < 0.0001$) indicate the significant difference between blocked and control studies by using the two-way ANOVA statistical test (GraphPad Prism 6.0). ns means nonsignificant.

The complexes **Tc1** and **Tc2** display a moderate hydrophilic nature as revealed by the partition coefficient (P) between 1-octanol and phosphate buffer pH 7.0 expressed as Log $P_{o/w} = 0.763 \pm 0.021$ and Log $P_{o/w} = -0.889 \pm 0.447$, respectively.

Aiming to assess the NET-recognizing ability of the benzylguanidine-containing compounds **L1** and metallated congener **Tc1**, we have performed a cell uptake study in SH-SY5Y cells. This cell line is a cloned subline of the neuroblastoma cells SK-N-SH that express monoamine transporters and were reported to take MIBG by an active mechanism.[46–48] The results from this study indicated a moderate **Tc1** uptake by SH-SY5Y cells that increased as a function of incubation time at 37°C (Figure 2). To assess the specificity of **Tc1** uptake, blocking studies with desipramine (DMI, 50 μM), a selective NET inhibitor, were carried out. A lower nonspecific uptake was found (~10–20%) suggesting that the mechanism of the uptake may be partially related with this transporter (Figure 2).

Encouraged by the cell uptake results, we have evaluated the biological behavior of **Tc1** in CD1 mice to assess its relevance as a potential radiotracer for myocardial imaging. The results of the biodistribution studies performed at 2-, 30-, and 60-min postinjection (p.i.) are presented in Table 1.

The complex showed a high heart uptake and a rapid clearance from the blood stream and the lung. After the initial high uptake (above 7.2% ID/g), a slight washout at the 1-h time point was found, but the radioactivity level remained persistently above 6% ID/g, resulting in a heart to blood ratio greater than nine. The heart uptake (6.3 ± 1.3% ID/g) is comparable to that observed for ^{99m}Tc-sestamibi, a radiopharmaceutical in clinical use for myocardial imaging, in the same animal model (8.0 ± 0.6% ID/g, 1-h p.i.).[49] However, the uptake in the heart is threefold lower than that of [^{125}I]MIBG at 1-h p.i. in mice.[50] It is also worth mentioning that **Tc1** presents a relevant accumulation in adrenals, a target tissue with high NET density.

To assess whether the moderate heart uptake is specifically related to NET, we have performed a blockade experiment by

Table 1. Biodistribution of **Tc1** in CD1 mice at 2, 30, and 60 min (% ID/g ± SD)

	% ID/g			Coinjection with nonradioactive MIBG
Organ	2 min	30 min	60 min	60 min
Blood	3.5 ± 0.7	0.66 ± 0.03	0.7 ± 0.3	0.75 ± 0.07
Liver	17.4 ± 7.5	22.4 ± 8.9	24.8 ± 5.6	21.98 ± 3.60
Intestine	4.5 ± 0.4	5.7 ± 0.7	6.7 ± 2.4	6.44 ± 0.62
Spleen	1.37 ± 0.08	0.9 ± 0.5	0.24 ± 0.01	1.47 ± 0.19
Heart	7.2 ± 1.5	7.8 ± 1.3	6.3 ± 1.3	3.89 ± 0.38
Lung	2.7 ± 0.7	0.9 ± 0.3	0.8 ± 0.4	1.30 ± 0.30
Kidney	67.9 ± 13.7	72.9 ± 4.5	73.7 ± 22.9	77.14 ± 5.33
Muscle	1.16 ± 0.05	0.7 ± 0.1	0.5 ± 0.2	0.31 ± 0.02
Bone	1.41 ± 0.06	0.6 ± 0.1	0.5 ± 0.1	0.35 ± 0.02
Stomach	1.2 ± 0.4	1.5 ± 0.7	1.4 ± 0.3	0.86 ± 0.37
Brain	0.18 ± 0.03	0.11 ± 0.07	0.10 ± 0.05	—
Thyroid	2.1 ± 0.2	1.3 ± 0.3	1.1 ± 0.5	—
Adrenal glands	6.0 ± 3.1	—	4.8 ± 3.5	1.3 ± 0.1
Ratio (heart/blood)	2.1	11.8	9.0	5.2
Ratio (heart/liver)	0.41	0.35	0.25	0.18
Ratio (heart/kidney)	0.11	0.11	0.08	0.05
Ratio (heart/lung)	2.8	8.6	8.9	3.0
Total excretion (% ID)	—	3.8 ± 1.1	5.2 ± 1.3	7.1 ± 0.8

coinjecting the mice with nonradioactive MIBG (1.0 mg/kg). In this group of animals, the heart uptake of **Tc1** was reduced by 38.2% at 60 min. This result suggests that the heart uptake mechanism of **Tc1** may be related to that of MIBG and is in agreement with the conclusion drawn from the cell uptake studies.

Aiming to confirm the hypothesis that the **Tc1** binding is specific and mediated by interaction of the guanidine moiety with the NET *in vivo*, we have also prepared **Tc2**, where the benzylguanidine pharmacophore has been replaced by a benzylamine group. In this case, we were not expecting a NET-mediated accumulation of radioactivity in the heart. The data of the biodistribution studies of **Tc2** confirmed our expectation, and a negligible heart uptake (0.56 ± 0.23% ID/g at 60-min p.i.; Table SI) was observed for this radioactive probe. This result is consistent with the fact that **Tc1** does not accumulate in the heart by a simple diffusion mechanism because **Tc2**, which displays similar physicochemical properties such as hydrophilicity and charge, has low accumulation in that organ.

Stomach uptake was very low for **Tc1**, indicating high *in vivo* stability of the complex, which was also confirmed by RP-HPLC analysis of urine and blood samples. However, a high liver (>24% ID/g) and kidney (>73% ID/g) accumulation was observed, which are considerably higher than the values found for [^{125}I]MIBG at 1-h p.i. (liver: 7.97 ± 0.97% ID/g and kidney: 3.21 ± 0.41% ID/g) in the same animal model [50]. With the aim of reducing the high level of radioactivity retention in the kidney, which is most likely related with tubular reabsorption, we have performed an additional set of biodistribution experiments in which Lys was coinjected with **Tc1** (Table SI). The coadministration of cationic amino acids (Lys and Arg) to inhibit renal accumulation of radiolabeled proteins and peptides is a well-established procedure, routinely used in the clinical setting.[51,52] Interestingly, coadministration of Lys did not significantly reduce kidney accumulation of the radioactive complex (61.45 ± 12.78% ID/g at 60-min p.i. for **Tc1** without coinjection of Lys and 56.19 ± 8.68% ID/g at 60-min p.i. for **Tc1** with coinjection of Lys), which seems to indicate that the electrostatic interaction between the positively charged complex and the negatively charged surface of the proximal tubule cells is not a major factor for renal radioactivity retention.

Conclusions

We have synthesized and assessed the pharmacokinetic profile of a new ^{99m}Tc(CO)$_3$-labeled benzylguanidine (**Tc1**), which is an MIBG derivative. Biodistribution studies in CD1 mice have shown that **Tc1** presents a moderate initial and persistent heart uptake, although with simultaneous high liver and kidney uptakes. The heart uptake was significantly decreased when coinjected with nonradioactive MIBG, suggesting a specific mechanism of accumulation mediated by the NET. Moreover, the blocking studies of the *in vitro* uptake of **Tc1** in cultured neuroblastoma cells suggested that the mechanism of heart uptake may be inhibited by desipramine, a specific NET inhibitor. Additionally, the biodistribution studies of **Tc2**, an analog complex where the benzylguanidine pharmacophore is absent, confirmed that **Tc1** does not accumulate in the heart by a simple diffusion mechanism but by a NET-mediated mechanism.

In conclusion, **Tc1** is a unique example of a ^{99m}Tc(CO)$_3$-labeled MIBG derivative with a reasonable heart uptake. Further work includes synthesis and investigation of novel derivatives in order to decrease kidney and liver uptakes, while maintaining the high persistent cardiac accumulation.

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Conflict of interest

The authors did not report any conflict of interest.

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